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Release and Degradation of Environmental DNA and RNA From Eels in Aotearoa New Zealand

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ABSTRACT

Environmental DNA (eDNA) has become a crucial tool for detecting rare species and monitoring biodiversity. However, the prolonged persistence of eDNA in water complicates the precise determination of an organism's location based on an eDNA signal alone. In contrast, environmental RNA (eRNA) degrades faster, potentially offering a more accurate detection proxy. To test this, we analyzed eDNA and eRNA release concentrations and decay rates from six longfin (*Anguilla dieffenbachii*) and six shortfin (*Anguilla australis*) eels under controlled conditions. Eels were placed in aquaria for 30 h and, after their removal, temporal water sampling was conducted over 7 days to assess the eels' eDNA and eRNA dynamics. Concentrations of eDNA and eRNA were estimated using validated droplet digital PCR assays for each species (*cytb* and 16S mitochondrial genes). Temporal eDNA and eRNA dynamics followed an exponential decay function over time, demonstrating a predictable decline in their concentrations. Moreover, higher decay rates of eRNA could represent a slightly more accurate proxy than eDNA for the location determination of rare species. Variability in the release and decay could be linked to the type of nucleic acid, marker genes, or eel species. Understanding these dynamics will help fine-tune detection models based on eDNA and eRNA.

1 | Introduction

Freshwater eels in Aotearoa New Zealand (AoNZ) play critical ecological roles as predators and prey, hold profound cultural significance in Māori traditions, and support economic activities through fisheries (McDowall 2011). However, freshwater eel populations have declined drastically in recent decades due to overfishing, habitat loss, and climate change (Dekker 2003; Arai 2014). Two main species of eel occur naturally in AoNZ freshwater streams and lakes (Cairns 1941): the longfin (LF) eel (*Anguilla dieffenbachii*), endemic to AoNZ, and shortfin (SF) eel (*Anguilla australis*), native to the region. These species face multiple threats, compounded by their catadromous life cycle (spawning at sea and growing in freshwater), which heightens their vulnerability to environmental stressors (Jacoby et al. 2015). The LF eel, classified as "endangered" by the International Union for Conservation of Nature (IUCN) (IUCN 2022), is widely distributed across AoNZ freshwater habitats in AoNZ and ranks among the largest species within the *Anguilla* genus (Todd 1980). Its populations have experienced a 75% decline in annual recruitment since commercial exploitation began in the 1970s, raising concerns about its long-term survival (Jellyman et al. 2000; Williams et al. 2017). The SF eel, listed as "near threatened" by the IUCN (IUCN 2022), has a narrower distribution in AoNZ but also occurs in other western South Pacific regions, such as Australia (Beumer and Sloane 1990) and is typically smaller than the LF eel. Population monitoring

is thus essential to warrant the conservation of these noteworthy species. Furthermore, understanding the movement of these eels throughout their life cycle is key to tracking their recruitment into AoNZ waters, ensuring their protection, and managing their stocks effectively.

LF and SF eels have a complex life cycle that exposes them to a variety of stressors throughout their life span due to the different habitats they must traverse during their migrations and the increasing anthropogenic pressures they face (Jacoby et al. 2015). Freshwater eels are diadromous, meaning they can migrate between freshwater and saltwater and exhibit a catadromous life cycle where juveniles develop in freshwater environments until sexual maturity. Once they have reached maturity, eels migrate to the open ocean for spawning and then die (Tesch 2003; Aida et al. 2003). Though some indications point to spawning regions within the westward-flowing South Equatorial Current (Miller et al. 2022, 2009; Kuroki et al. 2008; Aoyama et al. 1999; Jellyman 2003; Franklin et al. 2023), the exact locations of LF and SF eels spawning sites, as well as the distribution of their early life stages, remain one of the greatest unsolved mysteries among migratory species (Aida et al. 2003).

Traditional methods such as electrofishing and fyke nets have been used to collect and monitor eel populations in freshwater and estuarine systems, and midwater trawls have been employed to capture early life stages (leptocephali) in coastal and oceanic environments (Jellyman et al. 2000; Beumer and Sloane 1990; Miller et al. 2022). Satellite tracking has been used to comprehend the migration behavior of eels, with previous attempts thwarted by failed satellite transmission, detachment of high-tech tags, and individuals being preyed upon during their journey (Franklin et al. 2023; Koster et al. 2021; Schabetsberger et al. 2021; Jellyman and Tsukamoto 2010). These attempts have provided valuable data on eels' oceanic journeys but yielded limited insights on spawning migrations. Moreover, these methods can be stressful or lethal for the animals, require advanced skills, and often fail in obtaining accurate estimates of eels' abundance and distribution.

In recent years, molecular tools have revolutionized biodiversity surveys, and the use of environmental nucleic acids (eNAs), including environmental DNA (eDNA) and environmental RNA (eRNA), has become increasingly prevalent. Environmental nucleic acid monitoring is an indirect and non-invasive means of detecting the presence of organisms without the need to see or capture them (Taberlet et al. 2012; Bowers et al. 2021; Pauls et al. 2014). Additionally, the detection of aquatic species through waterborne eDNA/eRNA has proven to be highly sensitive and cost-effective, offering advantages for detecting rare, cryptic, and/or invasive species in marine and freshwater environments (Ficetola et al. 2008; Thomsen et al. 2012; Rees et al. 2014; Bohmann et al. 2014). Species-specific eDNA detection methods, using droplet digital or quantitative polymerase chain reaction (ddPCR/qPCR) analysis, have proven to be more effective than conventional techniques in freshwater systems for the detection of eels at low biomass densities (Itakura et al. 2019; Kasai et al. 2021; Thomson-Laing et al. 2021; Jannel et al. 2024). Despite relatively low levels of dissolved eDNA in open ocean settings, recent research has demonstrated the feasibility of using eDNA to detect Japanese eels (*A. japonica*) to elucidate potential spawning sites in the Pacific (Takeuchi et al. 2022, 2019a). Consequently, the use and development of new indirect methods, which are less disruptive, should be seen as a key direction for future studies aimed at monitoring the presence of these elusive species in various bodies of water. These methods could also be used to assist research in understanding the complete life history of LF and SF eels and be highly valuable to predict the impact of climate change on eel diversity and ecological distribution (Robinson et al. 2009; Egan et al. 2020) and to explore evolutionary factors that drive such migratory behaviours (Putman 2018).

Nevertheless, eDNA protocols can sometimes result in false positives due to various factors, such as the natural transport of long-persistence DNA molecules, genetic contamination, or sediment resuspension (Hansen et al. 2018; Cristescu and Hebert 2018), thus leading to misinterpretation of the location of the actual species found in a precise area at a specific time. The development of eRNA methods could provide a more accurate interpretation of biodiversity assessment results (Pochon et al. 2017; Kagzi et al. 2022). While eRNA is more labor intensive and costly to process, its theoretical transient persistence and ability to indicate the "active" proportion of a community could make its use valuable in minimizing false positive detections of non-local organisms while improving spatial and temporal resolution (Pochon et al. 2017; Cristescu 2019; Wood et al. 2020; Tsuri et al. 2021; Marshall et al. 2021; Jo et al. 2023; Jo 2023). While previous studies have started exploring eDNA/eRNA decay dynamics, including temperature effects on eel eNA degradation (Kasai et al. 2020), species-specific variations in these processes remain underexplored, particularly for LF and SF eels. As such, combining eDNA and eRNA traces may represent a powerful new approach for detecting rare and elusive species. However, an important and critical first step toward the application of these techniques in the field is to assess the "ecology" of eNAs by understanding their release and degradation dynamics in controlled environments (Cristescu 2019; Barnes and Turner 2016).

This study investigated eDNA and eRNA release and decay rates of LF and SF eels under controlled settings, using previously validated ddPCR assays for LF and SF eels (Thomson-Laing et al. 2021). The objectives were to compare the eDNA and eRNA release and decay rates of LF and SF eels in a controlled freshwater environment and to evaluate the temporal and thermal effects, species-specific rates, and the combined influence of single versus co-occurring species on eNA degradation. We hypothesized that (i) both eel species would release similar amounts of eDNA and eRNA in their surrounding environments with specimen sizes selected to be comparable; (ii) equivalent eNA decay rates would be observed between species or markers regardless of the presence of a second species, but that temperature would exert a significant effect on these rates; and (iii) eRNA would decay more rapidly than eDNA.

2 | Methods

2.1 | Study Location, Animal Collection, and Experimental Design

Wild LF eels were captured from the Brook Stream (Nelson, AoNZ, 41°16′ 39.244″ S, 173°17′ 22.872″ E) on the 1st of May 2023, using electro fishing equipment (Smith-Root LR-24, USA) and hand nets. Wild SF eels were captured on the same day

from a natural pond (Upper Moutere, AoNZ, 41°17′ 41.19″ S, 173°0′33.091″ E) using fyke nets left overnight. Based on morphological features, 12 eels ($n_{LF}=6$; $n_{SF}=6$) were identified and captured. Total body weight and length were measured, and health status was inspected before transfer into disinfected sealed fish bins containing filtered and UV treated tap freshwater (300 mJ/cm^2). Water temperature in the bins was maintained at 3°C lower than the river's and pond's respective temperatures to mitigate potential stress on the eels. Individuals were immediately transported to the Cawthron Aquaculture Park (41°11′ 31.495″ S, 173°20°54.683″ E) within 60 min from the field and placed into two separated clean acclimatation tanks (i.e., one for each species) containing filtered and UV treated tap freshwater, without feeding for 24 h prior to the decay rates experiment. This was done to (1) allow the eels to recover from the stress of capture and subsequent transportation, letting them acclimate to their artificial surrounding; and (2) facilitate the elimination of waste and potential environmental residue acquired before their immersion in the experimental aquaria. All equipment (e.g., fish bins, hand nets, aquaria) were thoroughly washed with a 10% bleach solution and rinsed with filtered (1 μm) and UV-treated water to prevent eNA contamination prior to receiving the eels. Each experimental aquarium was filled with non-recirculating UV-treated and filtered freshwater and equipped with cleaned air stones to keep the water oxygenated. To maintain a stable and similar-to-river temperature (15°C ± 1°C), aquaria were placed into large secondary containers (100 L cleaned plastic tanks) with temperature-controlled re-circulating water systems (Figure 1, Figure S1).

The experiment consisted of first placing the eels (individually or in combination) in their respective aquaria as to let them naturally shed nucleic acids. The experimental setup was as follows: three SF eels and three LF eels were placed individually in six separated aquaria (i.e., one eel per aquarium), hereafter referred to as 'single-species aquaria'. Additionally, three aquaria containing a combination of one SF eel and one LF eel of similar body size (Figure 1, Table S1), hereafter referred to as 'dual-species aquaria', were set up as described above. The rationale for the dual-species setup was to verify whether eDNA and/or eRNA degrades faster with increased organism density and microbial activity (Wood et al. 2020) and to test the specificity of ddPCR probes in multiplex assays. In our study, ddPCR was chosen due to its high sensitivity and specificity in detecting low-abundance DNA targets, which is particularly advantageous in eDNA and eRNA experiments (Thomson-Laing et al. 2021). One additional aquarium without eels served as a negative control tank (CT) during the trial. After 30 h in their respective aquaria, a sample was collected, and all animals were then removed using hand nets cleaned in a bleach solution and rinsed with Milli-Q (MQ) water (Millipore, Germany). Eels were then immediately and carefully transported and released back into the locations of capture. Temporal sampling of the water was then conducted regularly to assess decay rates over 7 days after removal (Figure 1).

2.2 | Sample Collection and Processing

One water sample (100 mL) was collected from each aquarium, from which both eDNA and eRNA were co-extracted. Sampling took place just before eel removal ($t=0$) and at the following timepoints after the eels were removed from their respective aquaria: $t=4$, 8, 12, 18, 24, 72, 120, and 168 h (Figure 1). Water samples were collected using sterile serological pipettes (50 mL), which were gently swirled ~10 cm from the aquaria bottom to ensure water mixing during sampling, followed by the transfer of each sample into a sterilized bottle. Samples were immediately filtered on 0.22 μm sterile cellulose membranes (MF-Millipore, Germany) using a low-pressure pump (Rocker Scientific, Taiwan). Filters were transferred into sterile bead tubes (ZR Bashing Bead Lysis Tubes; 2 mm Zymo Research, USA) and immediately frozen at −80°C until further processing of the eNAs to maintain and maximize the integrity of all

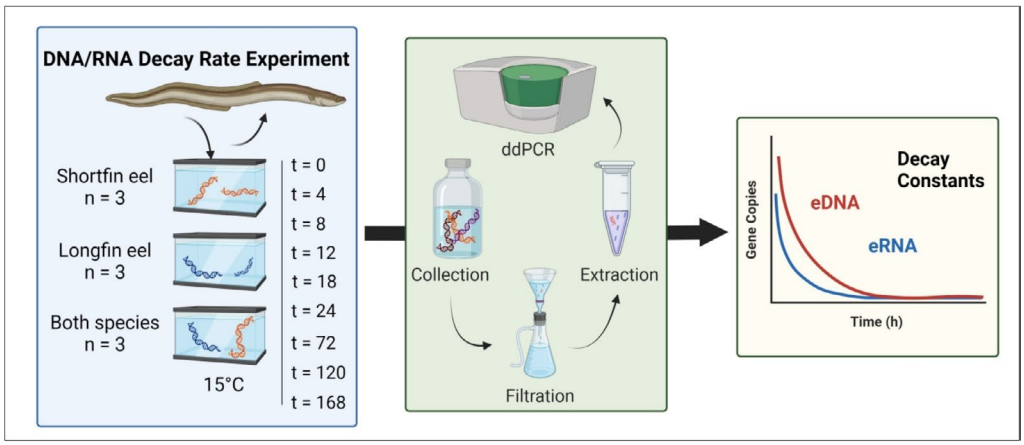

FIGURE 1 | Experimental setup. Each aquarium ($n=9$) contained 50 L of non-circulating filtered/UV treated water where eels (shortfin and longfin) were placed for 30 h before their removal. Water temperature was maintained at 15°C in all aquaria via recirculating flow in secondary containers. Water samples (100 mL) were collected at specific timepoints over a period of 7 days, with the presence of eels and after their removal, and were immediately filtered on 0.22 μm sterile cellulose membranes (MF-Millipore, Germany) and frozen. The main experiment ran at 15°C across all nine aquaria (three LF, three SF, and three dual species), reflecting typical river conditions. After eel removal ($t=0$), subsamples from the three dual-species aquaria were divided into six 2 L bottles, with three incubated at 30°C to simulate warm conditions and three at 4°C to mimic colder environments. DNA/RNA extraction and ddPCR analysis were performed to assess environmental nucleic acids' release and decay rates. Brief extraction steps can be found in Figure S2. The image was produced using Biorender.com.

genetic material (Gorokhova 2005; Wong et al. 2012). Between each sample, tweezers were ethanol-flamed, and filter holders were cleaned using a 10% bleach solution and rinsed with MQ water. Water samples (100 mL) from the CT were taken prior to the eels' introduction, at $t=0$ and $t=24$, and concurrently with experimental samples using identical, decontaminated equipment to assess possible contamination. One filter blank was processed at each time point using MQ water to confirm equipment decontamination. Physico-chemical parameters (temperature, pH, dissolved oxygen levels and ammonia concentrations) were monitored using multiparameter probes (YSI Professional Plus, USA) and ammonia/pH test kits (API Freshwater Master Test Kit, USA) every 5 h, while eels were present and at each sampling timepoint post-eel removal. The probes were rinsed with 10% bleach and in MQ water prior to immersion in the aquaria.

To assess the potential effect of temperature on eNA degradation, 2 L of well-mixed water in duplicate from each dual-species aquarium ($n=3$) was isolated into six sterilized bottles at $t=0$. For the duration of the experiment (7 days), half of the bottles (one from each tank) were placed in an oven at 30°C, and the other half was stored in a cold room at 4°C, while the aquaria remained at 15°C. Water samples (100 mL) were filtered at timepoints following the same procedure as described above.

2.3 | DNA and RNA Extractions

Filtered water samples were extracted in the Molecular Suite Lab of the Cawthron Institute, Nelson, AoNZ. To avoid cross-contamination, each step of the molecular analysis took place in a separate sterile laboratory room with sequential workflows. DNA and RNA co-extraction was similar to the protocol described in Wood et al. (2020)., with minor modifications. Each room was equipped with UV sterilization switched on for a minimum of 15 min before and after each use to eliminate possible DNA/RNA contamination from previous work. For each sample, frozen filters contained in the ZR BashingBead Lysis Tubes were split into pieces directly within the tubes using sterile tweezers. DNA/RNA shield solution (600 μL) from the Quick DNA/RNA MiniPrep Plus Kit (Zymo Research, USA) was added to each tube. Samples were then homogenized by bead beating (1500 RPM, 2 min). Proteinase K (30 μL) and Proteinase K digestion buffer (60 μL) were added, followed by a 30 min incubation at room temperature. The aqueous phase of the sample containing the nucleic acids was separated by centrifugation (12,000 × G, 30 s, 20°C) before adding the kit's lysis buffer (690 μL). DNA and RNA were then co-extracted using Quick-DNA/RNA Miniprep Plus Kit (Zymo Research, USA), following the manufacturer's protocol. DNA and RNA were resuspended in 30 μL nuclease-free water and stored frozen (−20°C for eDNA and − 80°C for eRNA).

2.4 | Complementary DNA Synthesis for eRNA Samples

As described in Langlet et al. (2013)., trace DNA molecules carried over in RNA extracts were eliminated by two sequential DNase treatments. Polymerase chain reactions (PCR) were then performed by amplifying the universal vertebrate DNA marker (mitochondrial 12SV5) (Riaz et al. 2011) to confirm the absence of DNA in each RNA sample eluents. One PCR negative and one positive control (sterile water and DNA extracted from LF and SF tissue, respectively) were included in each PCR run. Each PCR reaction (total volume = 25 μL) included primers (forward and reverse, 454 nM), 1 × Mytaq red mix (Bioline, Canada), and 2 μL of DNAse-treated RNA sample. The thermocycling conditions were as follows: 95°C (5 min) for initial denaturation, 30 cycles of 95°C (30 s), 54°C (45 s), 72°C, then a final step of 72°C (5 min) for enzyme deactivation. When DNA was still present in RNA samples as indicated by a positive PCR amplification of the RNA sample, a second round of DNase treatment and PCR check were performed. Isolated total RNA was quantified in each sample using a Qubit fluorometer (Invitrogen, USA) and a RNA Quantification Broad Range kit (Invitrogen, USA) to allow measurement of RNA concentration for the complementary DNA (cDNA) synthesis. The DNase-treated RNA was then transcribed into cDNA using random hexamer primers (Thermo Fisher scientific, USA) and SuperScript III Reverse Transcriptase (Langlet et al. 2013) (Life Technologies, USA). Blanks were included at each stage: filtration blanks (MQ water), DNA digestion blanks (nuclease-free water), and reverse transcription blanks (no-template controls). Aliquots were stored frozen (−80°C for DNAse-treated RNA backup samples and − 20°C for cDNA) until further analysis. The cDNA products are hereafter referred to as eRNA.

2.5 | Droplet Digital Polymerase Chain Reaction

Droplet digital PCR was performed using the Biorad Q×200 ddPCR system composed of an automated droplet generator setup with droplet oil for probes, a PCR thermocycler and a droplet reader (Q×200 Droplet Digital PCR System, Biorad, USA). Copy numbers (per μL) of the mitochondrial 16S ribosomal RNA (16S rRNA) gene and mitochondrial *cytochrome b* (*cytb*) gene were measured in all samples using primers and probes previously validated (Thomson-Laing et al. 2021) for SF and LF, respectively. Primers were designed to specifically target regions with the greatest interspecific variability among eel species identified in AoNZ (Thomson-Laing et al. 2021). The following primers and probe targeting the mitochondrial 16S ribosomal RNA (16S rRNA) gene of SF eels were used: forward primer (A.aust16S-F: 5′–CCC AAA AGC AGC CAC CTG–3′), reverse primer (A.aust16S-R: 5′–AGG GGG TGG GGA GTT TAT TA–3′), and primetime probe (A.aust16S-P: 5′–/56-FAM/AAA GAA AGC/ZEN/GTT AAA GCT CCG A/3IABkFQ/–3′). For LF eels, the mitochondrial *cytochrome b* (*cytb*) gene was targeted: forward primer (A.dieffCytB-F: 5′–GAT TCT TCG CAT TCC ACT TCT TA–3′), reverse primer (A.dieffCytB-R: 5′–GGA CTT TGT CTG CGT CAG AGT TT–3′), and primetime probe (A.dieffCytB-P: 5′–/5-HEX/TCC TAC ATG/ZEN/AAA CAG GAT CAA GCA ATC CA/3IABkFQ/–3′). Prior to amplification, the extract products (i.e., eDNA and eRNA samples) were diluted 1 in 10 as preliminary experiments indicated inhibition effects. Each ddPCR reaction (total volume = 22 μL) included primers (forward and reverse, 454 nM), probes (FAM and HEX, 454 nM), 1 × ddPCR SuperMix for probes (Bio-Rad, USA), 5 μL DNA/eDNA, and sterile water for the negative control. The multiplex ddPCR mixture was then combined with 70 μL of BioRad droplet oil for probes and partitioned into approximately

20,000 nano-oil droplets by the ddPCR droplet generator Q×200 (Biorad, USA). The nanodroplet emulsion (40 μL) was transferred and amplified in a PCR plate using the following cycling protocol: 95°C (10 min) for initial denaturation, 45 cycles of 94°C (30 s), and 59°C (1 min), then a final step of 98°C (10 min) for enzyme deactivation. For each run, one negative methodological control (i.e., CT tank sample), one ddPCR negative (nuclease-free water), and positive controls (LF and SF extracted tissue DNA) were included to monitor possible contamination. The limit of detection was set at 0.1 copy per μL for eDNA samples and 1.70 copies per μL for eRNA samples due to non-specific products (e.g., primer-dimers) from cDNA synthesis. The PCR setup and template addition were undertaken in laminar flow cabinets with HEPA filtration.

2.6 | Decay Rate Constants, Half-Lives, and Data Analysis

All statistical analyses were conducted using R version 4.1.3 (R Core Team 2022). Decay rate calculations were similar to the ones performed by Wood et al. (2020). and Lance et al. (2017)., established by fitting a monophasic exponential decay model to all raw data:

$$N(t) = N_0 e^{-\lambda t} \quad (1)$$

Where $N(t)$ is the concentration (copies per μL) of LF or SF eDNA or eRNA at time t, N_0 is the concentration of LF or SF eDNA or eRNA at $t = 0$, and λ is the decay rate constant. Decay rate models were fitted using 'easynls' package in R (Kaps and Lamberson 2004). As decay initial concentrations (i.e., detected concentrations after 30 h with the presence of eels) of eDNA and eRNA varied among aquaria, N was transformed to $\hat{N}$, which is the eNA concentration relative to the maximal concentration value (copies per μL) of each aquarium (Kasai et al. 2020). $\hat{N}$ was then fed back into Equation (1) to access the final values of λ. Half-lives were calculated using the following formula:

$$t_{1/2} = \frac{ln(2)}{\lambda} \quad (2)$$

Where the half-life $t_{1/2}$ is the time required for the eNA sample concentration to decrease to one-half of its initial value, and λ is the estimated decay rate constant of interest (expressed in h^{-1}).

For initial released concentrations comparisons, eNA concentrations at $t = 0$ h were log-transformed to facilitate visualization and mitigate heteroscedasticity. To analyze the effects of nucleic acid type (eDNA/eRNA), species (longfin or shortfin eel), biomass, treatment (isolation or combined) on the initial concentrations, and temperature on decay rates of eDNA and eRNA, linear mixed models (LMMs) and generalized linear mixed-effects models (GLMMs) were generated, respectively, using lme4 and lmerTest R packages (Bates et al. 2015; Kuznetsova et al. 2017). Aquarium ID was included as a random effect to account for variability between aquariums that might influence eNA concentrations. For eDNA and eRNA concentration, the response variable (log-transformed eNA concentration) was modeled using a normal distribution: lmer log eNA concentration ~ biomass + NA type (eDNA/eRNA) * species (LF/SF) + treatment (single/dual species aquaria) + (1 aquarium ID). For the decay rate constants (λ), a Gamma distribution was used due to the non-negative nature of the data: glmer (decay rate (λ) ~ NA type (eDNA/eRNA) + species (LF/SF) + treatment (single/dual species aquaria) + temperature (4°C/15°C/30°C) + (1 aquarium ID)). Model selection was performed based on Akaike Information Criterion (AIC) values. The evaluation of each model's fit was carried out using residual diagnostics implemented through the R package DHARMa (Hartig et al. 2024). The significance level was set at = 0.05.

2.7 | Ethics Statement

This study was approved by the Cawthron Institute and the Nelson Marlborough Institute of Technology Animal Ethics Committee (protocol number: AEC-2023-CAW-02) and was conducted in accordance with the guidelines of the New Zealand Council for the Care of Animals in Research and Teaching. Authors complied with the ARRIVE guidelines.

3 | Results

3.1 | Eels Measurements and Physico-Chemical Parameters

The mean (± SD) mass of captured eels used in the experiment was 615 ± 307 g for LF and 701 ± 253 g for SF (Table S1). The mean length was 520 ± 91 mm for LF and 570 ± 82 mm for SF. The temperature, ammonia concentrations, dissolved oxygen levels, and pH in each aquarium remained relatively constant throughout the experiment (Figure S3). Dissolved oxygen levels slightly dropped (from 11.2 to 9.8 $mg.L^{-1}$ on average) after eels' removal, probably as a result of the air stones being switched off, but remained at saturation levels.

3.2 | Quantification of Environmental DNA and RNA Shedding

No amplification occurred in any of the control tank samples, filter blanks, cDNA synthesis, or PCR negative controls throughout the duration of the experiment. In this study, 150 samples were successfully co-extracted ($n = 150$ eDNA samples and $n = 150$ eRNA samples). All eDNA extracts and 134 eRNA extracts synthesized to cDNA (i.e., excluding control tank eRNA, pre-eel introduction eRNA and four eRNA samples that failed due to handling in the cDNA synthesis step) were processed for ddPCR. For both species, primer pairs successfully amplified their targeted gene fragments/species in ddPCR multiplex runs. DNA was still present in 31 eRNA extracts; therefore, a second DNAse sequential treatment and PCR check were performed to ensure complete elimination of DNA in these samples. On average, maximum concentrations of eNAs were observed right before removing the eels from the aquaria ($t = 0$ h) for both species (Figure 2), but high levels of eNAs were also observed 4 h post eel removal. Released eNA mean concentrations were significantly affected by SF eels in single species aquaria ($t = 10.262$, $p = < 0.001$) and dual species aquaria ($t = 10.582$, $p < 0.001$), while the effect of LF eels was not significant ($t = 1.767$, $p = 0.1$)

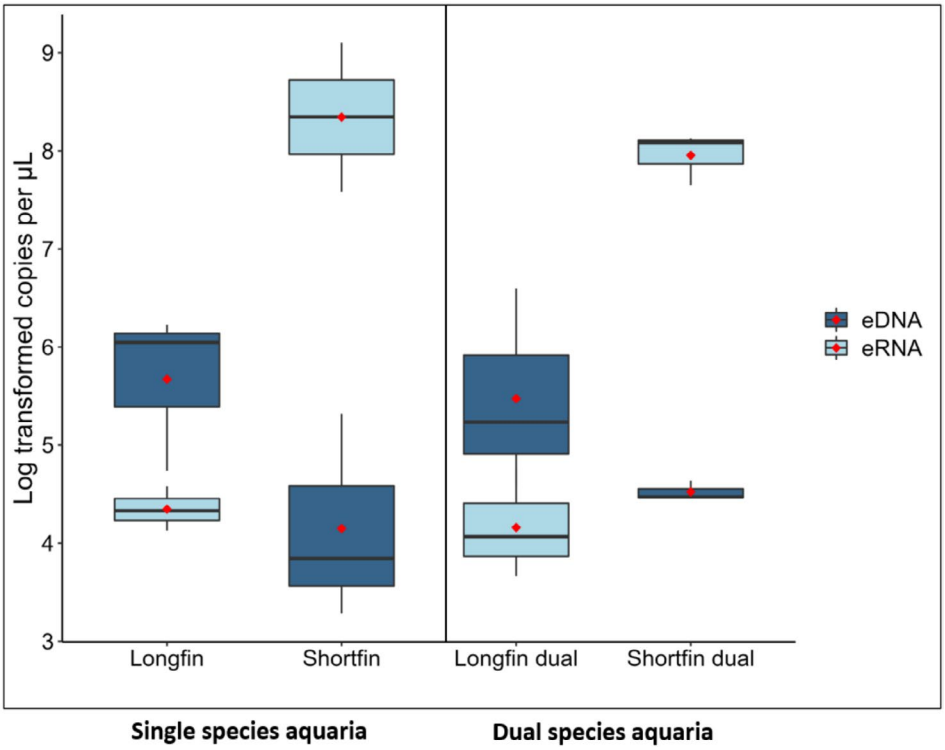

FIGURE 2 | Initial detected concentrations of eDNA and eRNA at $t = 0$ h (log-transformed copies per µL at N_0) from individual longfin and shortfin eels (single-species aquaria), and aquaria containing both LF and SF eels (dual-species aquaria). Boxplot of paired samples (eDNA and eRNA co-extracted from the same water sample) were superimposed to facilitate reading. The median is shown by the solid line across the box while the mean is indicated by the red diamond. Whiskers show minimum (lower) and maximum (upper) values.

(Table S2). Overall, concentrations varied significantly depending on eNA type ($t = 3.661$, $p < 0.01$) and biomass ($t = 3.136$, $p < 0.05$). Specifically, for LF eels, eDNA was detected on average at higher concentrations than eRNA in both single and dual-species aquaria. In contrast, for SF eels, the opposite effect was observed, with very high levels of identifiable eRNA compared to eDNA (Figure 2). Longfin eels yielded on average 3.5 times more copies of eDNA than eRNA, while SF eels yielded on approximately 54 times more copies of eRNA than eDNA at $t = 0$ h (Figure 2).

3.3 | Decay Rates

The eDNA and eRNA concentrations of each aquarium exhibited exponential degradation that could be fitted to monophasic exponential decay models (Figure 3; Figures S4 and S5). R^2 values of the fitted curve ranged from 0.94 to 0.99 for the single and dual-species aquaria, indicating that model-estimated curves strongly fit the data. The mean decay rate constants λ varied significantly among treatments (including temperatures) and species from 0.01 to 0.23 h^{-1} for eDNA and from 0.01 to 0.51 h^{-1} for eRNA (Table 1, Figures S5–S6, Table S3). Decay dynamics were modeled using a generalized linear mixed model (GLMM) with a Gamma distribution, where aquarium ID was included as a random effect to account for variability between aquaria. The model revealed that decay rates were significantly influenced by nucleic acid type and temperature, but not by species or tank composition. Specifically, eRNA degraded significantly faster than eDNA across all treatments ($t = -4.011$, $p < 0.001$), reflecting its lower stability and higher susceptibility to degradation. Temperature also had a significant positive effect on decay rates, with higher temperatures accelerating degradation ($t = 3.110$, $p < 0.01$). For instance, at 30°C, eRNA became undetectable after 24 h, while eDNA remained detectable at low concentrations up to 168 h. Conversely, at 4°C, both eRNA and eDNA degraded more slowly, remaining detectable at $t = 168$ h. The decay rate constants ranged from 0.092 h^{-1} to 0.133 h^{-1} for the high-temperature treatment and from 0.012 h^{-1} to 0.018 h^{-1} for the low-temperature treatment (Figure S5). The model further demonstrated that species (i.e., LF vs. SF eels) and tank composition (single vs. dual-species) did not significantly affect decay rates (all > 0.2). However, in dual-species aquaria, shortfin eDNA decayed faster than in single-species aquaria (Figure 3d), while no such differences were observed for longfin eels.

Half-life decay times, presented in Table 1, ranged from 9.0 h to 33.9 h for eDNA and from 4.8 h to 9.5 h for eRNA. Longfin and SF eDNA were still detected at $t = 168$ h post-eel removal, while LF eRNA was detected until $t = 72$ h, although at very low levels, and SF eRNA was detected until $t = 168$ h.

4 | Discussion

The main objective of this study was to assess the release and decay dynamics of nucleic acids in freshwater for two species of AoNZ eels. The first hypothesis stipulated that both eel species would release similar amounts of eDNA and eRNA, given similar mass, in their surrounding environments. However,

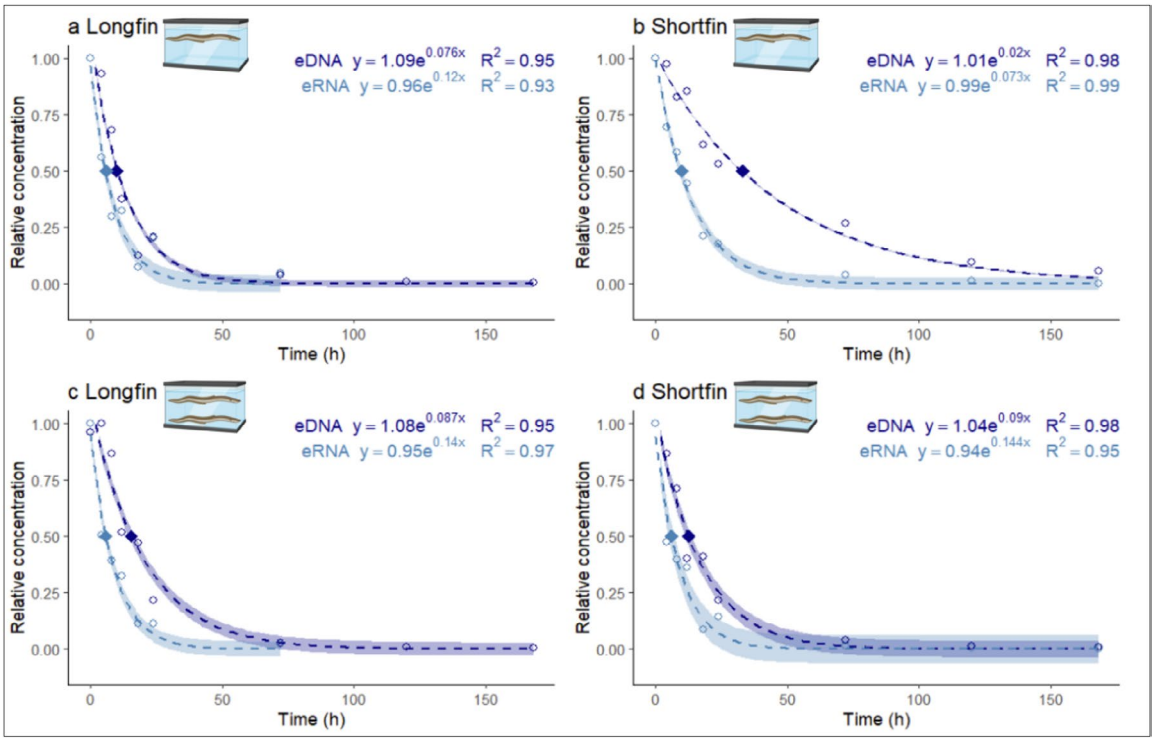

FIGURE 3 | Average temporal change in eDNA and eRNA concentrations post-eel-removal for longfin (left) and shortfin (right) eels in two distinct treatments (single-species aquaria: a, b; or dual-species aquaria: c, d, respectively). Different colors indicate different eNAs (dark blue for eDNA, light blue for eRNA). Equations show the rate of exponential decay after applying the decay model $\hat{N}(t) = \hat{N}_0 e^{-\lambda t}$ to all raw data. Dashed lines represent the best fit exponential regression curve to the fitted decay model. R^2 values indicate the closeness of fit of relative-calculated data to the fitted decay model. Diamonds represent the half-life decay time ($t_{1/2}$) of eDNA and eRNA. Smoothed curves represent mean standard error for each curve.

after 30 h of immersing the animals in various aquaria, each species released variable amounts of eNAs that were easily detectable using previously validated ddPCR assays (Thomson-Laing et al. 2021). Although the total average mass and length of the individual eels were similar across both species, higher concentrations of eDNA were detected for LF compared with SF eels. Additionally, the presence of another eel species in the same aquarium did not significantly influence the eDNA shedding concentrations compared to single eel species in isolation, which supports results observed previously for two species of carangid fishes (Murakami et al. 2022). Several explanations may account for these discrepancies including physiological factors such as differential stress responses or metabolic rates; for example, differential stress levels that each species experienced prior to or during the experiment (e.g., LF shedding more DNA with higher stress levels due to electrofishing despite mitigation efforts), the general metabolic activity (Wood et al. 2020; Sassoubre et al. 2016; Thalinger et al. 2021), and/or differences in skin mucus shedding amounts and their associated e DNA concentration. The composition of skin mucus can vary depending on the species, and previous work on the genus *Anguilla* demonstrated significant differences in skin mucus functional composition among eel species, including *A. dieffenbachii* and *A. australis* (Tsutsui et al. 2015). More specifically, the 19 species investigated in the latter study exhibited different C-type lectin-encoding genes, suggesting that *Anguilla* species secrete different types of mucus that are adapted to the microflora of their environment. Therefore, quantifying the amount of DNA in eels' mucus (both intracellular and extracellular) with targeted genes used in the present study (*cytb* and 16S rRNA) could offer valuable information on the DNA concentrations that each species release in this case. More generally, while only one study demonstrated that a higher activity of fish increases the amount of shed eDNA (Thalinger et al. 2021), further investigation is required to establish the levels of eDNA released in different sample types (e.g., tissue, scales, mucus, feces), and to gain a better understanding of how fish physiologies, metabolic rates, and dietary habits impact the release of eDNA (Takeuchi et al. 2019b).

Observed differences in eDNA concentrations can be considered negligible compared to the variability of eRNA initial concentrations obtained in the present study. Initial SF eRNA concentrations were significantly higher than SF eDNA concentrations, whereas an inverse trend was observed for LF eels. Concentrations of SF eRNA were on average 54 times higher than SF eDNA and 14 times higher than LF eDNA. Meanwhile, LF eRNA was on average five times lower than LF eDNA. In light of these findings, other potential explanations can be formulated. Firstly, differences in eRNA manipulation from the extraction step to the quantification step, and particularly the conversion of RNA to cDNA involving the use of reverse transcriptase, may lead to artefactual products. This enzyme, unlike DNA polymerase, lacks proofreading activity which could result in biased cDNA sequences after cDNA synthetisation (Laroche et al. 2017). Degradation processes could also impact the final detection as RNA is particularly labile and exposed during these stages (Wood et al. 2020). While differences may come from cDNA synthesis, the identical treatment of samples throughout the experiment makes this unlikely. The most likely hypothesis to consider is related to the targeted assay genes. Both species

TABLE 1 | Mean decay rate constant (λ, mean ± SE) and half-life decay time ($t_{1/2}$) of eDNA and eRNA for LF and SF in two distinct treatments (Single-species: One eel per aquarium; Dual-species: One eel of each species per aquarium).

Species and targeted gene	Type of eNA	Treatment	Mean decay rate constant λ (h^{-1})	$t_{1/2}$ (h)
Longfin	eDNA	Single-species 15°C	0.076 ± 0.014	9.2
cytb		Dual-species 15°C	0.087 ± 0.029	8
		Dual-species 4°C	0.013 ± 0.004	53.3
		Dual-species 30°C	0.123 ± 0.031	5.6
	eRNA	Single-species 15°C	0.120 ± 0.038	5.8
		Dual-species 15°C	0.140 ± 0.033	5
		Dual-species 4°C	0.018 ± 0.006	38.5
		Dual-species 30°C	0.133 ± 0.070	5.2
Shortfin	eDNA	Single-species 15°C	0.020 ± 0.003	33.9
16S rRNA		Dual-species 15°C	0.090 ± 0.036	7.7
		Dual-species 4°C	0.012 ± 0.009	57.8
		Dual-species 30°C	0.092 ± 0.052	7.5
	eRNA	Single-species 15°C	0.073 ± 0.028	9.5
		Dual-species 15°C	0.144 ± 0.062	4.8
		Dual-species 4°C	0.016 ± 0.005	43.3
		Dual-species 30°C	0.099 ± 0.038	7.0

exhibit high sequence similarities (97% and 94% for 16S rRNA and *cytb* genes, respectively). Primers and probes were developed using different gene regions for each species (16S rRNA for SF and *cytb* for LF) to avoid cross-reactivity (Thomson-Laing et al. 2021). While both targeted genes were mtDNA genes, different target regions (16S rRNA and *cytb*) might have yielded variable amounts of recovered eNAs (Marshall et al. 2021). Recent work by Marshall et al. (2021). demonstrated different eRNA shedding concentrations from the same species when considering eNAs type and origin highlighting that mitochondrial rRNA yielded concentrations of *Dreissena* mussels were > 1000 times higher than their mitochondrial DNA counterparts, and > 50 times higher than mitochondrial mRNA (Marshall et al. 2021). Hence, rRNA genes are expected to exist in higher concentrations than mRNA in environmental samples. Consequently, the ability to detect eRNA could largely depend on the specific RNA type and might explain the observed differences in eRNA concentration between markers and species in the present study.

The second hypothesis was that equivalent eDNA decay rates would be observed between species regardless of the number of isolated or co-occurring species in each aquarium. All amplified eNAs followed an exponential decay pattern for both species after eel removal, confirming numerous observations reported in previous studies (Kagzi et al. 2022; Wood et al. 2020; Scriver et al. 2023; Barnes et al. 2014; Qian et al. 2022). Overall, estimated degradation rates for eDNA using monophasic exponential decay models (0.022 to 0.087 h^{-1}) were within the lower half of the reported eDNA rates for freshwater and marine fishes for which Scriver et al. (2023). compiled a review of decay constants ranging from 0.013 to 0.697 h^{-1}. Furthermore, these rates, although slightly higher, were within a similar range of those reported for the Japanese eel *A. japonica* (0.017 to 0.040 h^{-1}), from temperature treatments ranging from 10°C to 30°C (Kasai et al. 2020). However, in this study, different eDNA decay rates were obtained between species and treatments at 15°C, as individual SF eDNA decay rates were notably lower than LF eDNA and SF eDNA from dual tanks. The observed disparities in SF eDNA decay rates depended on the treatment (single-species or dual-species aquaria), thus rejecting the second hypothesis and providing valuable evidence to support the assumption proposed by Wood et al. (2020). The latter study suggested that combining species leads to faster degradation of eDNA because of increased organism density and therefore microbial activity. Bacterial extracellular nuclease activity, which can play a crucial role in eDNA degradation through enzymatic hydrolysis (Lance et al. 2017; Zulkefli et al. 2019) was not measured in the present study. It is worth noting that the experimental conditions used here do not mirror natural environmental conditions where higher biological activities will contribute toward further accelerated degradation of eNAs (Bowers et al. 2021; Scriver et al. 2023). Regarding the differences between LF and SF eDNA differing degradation rates, the most likely explanation is that the nature of floating DNA could differ depending on the species, with mucus type possibly influencing the state of intra or extracellular DNA consequently detected. As described above, differences could also be explained by the use of different genetic markers. In addition to the dual-species effect, the temperature effect was assessed in our experiment. The rates measured during our add-on temperature experiment confirm the results obtained by Kasai et al. (2020), indicating a reduction in eNA degradation at low temperatures and an increase at high temperatures. Comparing the rates obtained across a wide range of temperatures (from 4°C to 30°C) is ecologically relevant to the studied species, as eels encounter diverse temperature conditions during various life stages. In freshwater environments, eels migrate both up and down deep and shallow streams, which may result in significant temperature fluctuations in their juvenile and adult life stages (Cairns 1941; Tesch 2003; Aida et al. 2003). Additionally, during their journey from the AoNZ waters to the warm and tropical environments of the Pacific islands, eels vertically navigate the open ocean water column

(Franklin et al. 2023; Jellyman and Tsukamoto 2010) releasing eNAs at different depths encompassing the aforementioned temperature range. It is important to acknowledge that within natural ecosystems, specific local environmental factors such as pH, temperature, salinity, light, and microbial communities can further facilitate the degradation of eNAs, thereby leading to differing decay rates compared to those observed under experimental conditions. However, this work provides a strong baseline for future investigations.

The third hypothesis was that eRNA would decay more rapidly than eDNA. As expected, mean decay rates were higher for eRNA than for eDNA. This was true regardless of the targeted gene and initial concentrations. Detection limits were attained later than expected for eRNA (up to $t = 168$ h) but in a similar range to previous reports (Jo et al. 2023). Higher concentrations of initial SF eRNA likely explain the long detection time ($t = 168$ h) compared with the relatively swift ($t = 72$ h) disappearance of LF eRNA in the 15°C aquaria. Additionally, as explained above, differences in eRNA origin (rRNA vs. mRNA) could also play a significant role in the observed detection time. In comparison with published studies conducted on freshwater invertebrates (*Dreissena* mussels (Marshall et al. 2021), *Daphnia* water flea (Kagzi et al. 2022)) vertebrates (*Danio* zebra fish (Jo et al. 2023)), which displayed eRNA decay rates ranging from 0.02 to 0.9 h^{-1} in similar temperature and pH conditions used here, the eRNA decay rates of *anguillid* eels were, on average, higher (0.073–0.144 h^{-1}). However, two other studies in seawater assessing invertebrate eRNA decay rates (malacostraca, polychaete, ascidian) (Wood et al. 2020; Qian et al. 2022) reported even higher rate constants, ranging between 0.16 and 1.68 h^{-1}. As noted earlier, salinity may modulate eRNA stability—a factor not tested here but relevant to the diadromous life history of eels. While eDNA studies have yet to provide firm evidence for salinity's effect, its influence on eRNA decay also merits further investigation (Scriver et al. 2023), particularly for diadromous organisms occurring across salinity gradients.

In the present study, the half-life ($t_{1/2}$) of decaying eNA ranged between 8 and 33.9 h for eDNA and from 4.8 to 9.5 h for eRNA at 15°C, indicating faster degradation (2.2 times on average) for eRNA. Moreover, temperature has been shown to have a significant effect on degradation dynamics, showing that RNA degradation can be slower than that of DNA in a colder environment, although the mean degradation of RNA was always faster than that of DNA. Environmental RNA is assumed to degrade faster because of its single-stranded structure (Eigner et al. 1961), sensitivity to oxidation from reactive oxygen species, and susceptibility to spontaneous cleavage of its phosphodiester bond (Fabre et al. 2014). Conversely, the double-stranded configuration of DNA is thought to reduce the susceptibility to hydrolytic processes, which may partly explain the difference in decay rates observed, albeit not 50 times greater, as suggested previously (Eigner et al. 1961). To address ecological questions where detection levels can be lowered (e.g., high dilution factors in open environment settings, low organism density), the higher concentrations and increased decay rates observed from SF eRNA could represent a more accurate proxy than eDNA for the detection of rare and mobile aquatic vertebrates. But these considerations must be taken with a degree of caution since the degradation of RNA can be lower than that of DNA under different temperature conditions. This makes conclusions more difficult to reach. The parameterization of models using eNAs decay rates must therefore take into account the specific zone of the water column being studied, in which many parameters vary, not least temperature.

The concentration of eNAs released into water by an organism is an important factor to consider when it comes to detecting animals in their natural environment. Findings from the present study demonstrated that eNAs concentrations may largely be dependent on the species as well as the targeted gene of interest. The development of an appropriately sized marker (Jo et al. 2017) able to accurately differentiate eel species using a single multiplex ddPCR assay (von Ammon et al. 2022) could represent a valuable alternative (i.e., lower costs and time) to assess the presence/absence of eels in an open environment, with higher accuracy (Rees et al. 2014; Jo et al. 2017; Taberlet et al. 2018). Analyzing eDNA and eRNA together offers complementary insights into species presence and activity. While eDNA provides a persistent signal of historical presence, eRNA's rapid degradation could indicate recent activity, potentially distinguishing between nearby and distant populations when detected concurrently. Moreover, using eNAs as an accurate proxy of the presence, abundance, and location of targeted organisms requires an understanding of the rates at which eNAs are released and decay (Scriver et al. 2023). Indeed, transport of persistent genetic material in water can bias organisms' distribution estimates due to possible diffusion through flows and currents over long distances. The ability to relate eNA information to its original source is therefore limited to our understanding of temporal and physico-chemical factors which influence eNA dynamics and detectability (Tsuri et al. 2021; Scriver et al. 2023; Moushomi et al. 2019). In the context of this study, and to cover temperatures and environments encountered by eels throughout their life cycle, an optimal experiment would involve the use of seawater with mature and larval individuals and temperatures corresponding to the different depths of water columns from AoNZ to the western South Pacific region. Such endeavor was beyond the scope of this work, both ethically and logistically (Takei and Hwang 2016), but should be further investigated to provide refined measures of eel eDNA and eRNA release and decay rates for accurate eNAs particle tracking models in the marine environment. Nevertheless, the presented results represent a first important step in the understanding of eNAs ecology and fate in an aquatic ecosystem.

5 | Conclusion

The utilization of eDNA and eRNA has become increasingly popular for the detection of rare and cryptic species in biodiversity monitoring. However, the ecological implication of using both types of NAs has never been investigated for diadromous fish. This study focused on measuring the amount of eDNA and eRNA released by and the degradation rates from two AoNZ eel species (*A. dieffenbachii* and *A. australis*) under controlled conditions. Our findings highlighted the variability in eNAs release and decay between nucleic acid types, marker genes, species, and temperature, emphasizing the need to consider factors such as fragment length, genomic source, as well as the physiological and ecological attributes associated with each eel

species. Environmental RNA degraded faster than eDNA when observed in similar conditions, but the transient nature of this molecule is questionable in our experiment, since certain factors (e.g., species, temperature, and marker) may promote prolonged persistence of eRNA. Understanding the principles of gene-dependent detection and quantification is crucial for the advancement of eNA methodologies and their application in aquatic ecosystems for sampling strategies. As such, future studies should focus on the development of species-specific primers and probes targeting a single gene marker to refine eel detections within any aquatic setting. Furthermore, experimental trials in seawater settings will enable refined detection by limiting cross-reactivity with other eel species in the western South Pacific region that would be key to tracing LF and SF eel migrations routes. In this regard, the use of precise decay rates would play an important role in the calibration of particle tracking models investigating eDNA and eRNA dispersal to better understand eels' movements.

Author Contributions

X.P., A.J.M.S., and A.C.-P. were involved in developing the experimental design. X.P. and A.C.-P. conducted the aquaria experiments. A.C.-P. and H.G.H. conducted all molecular lab work, collected, and analyzed the data. A.C.-P. wrote the first draft of this manuscript. X.P., A.J.M.S., H.G.H., G.T.L., and T.M. provided feedback for the analysis and contributed to the revisions.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data and metadata generated for this study can be sent via email or are available online on the following link: https://github.com/alexandreche/Data_Eels_eDNA_eRNA_Che_et_al.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.